Claim 32 was also amended to particularly point out that the sensing moiety is capable of binding with the analyte. This amendment is supported by the specification at p. 7, ll. 16-18. This amendment does not constitute new matter. A marked up copy of the claims, showing the changes made by the present amendment, is attached to this document.

II. RESPONSE TO THE OFFICE ACTION

A. Status of the Claims

The Examiner imposed a seven-way restriction requirement in a Office Action dated October 24, 2002. Applicants elected, with traverse, claims 32-38. Claim 32 was amended. The restrictions requirement was made final in the present Office Action and claims 1-31 and 39-43 have been cancelled in the present Amendment. Applicants reserve their right to pursue these claims in divisional or continuation applications. Claims 32-38 are pending in the application. Claim 32 has been amended by the present Amendment.

B. Rejection under 35 U.S.C. § 112

Claims 32-38 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, he Examiner alleges that claim 32 is vague and indefinite because it is unclear how the sensing moiety is related to the analyte. Applicants respectfully traverse. Claim 32 has been amended to more specifically point out that the sensing moiety is capable of binding with the analyte.

The Examiner also alleged that claims 32-34 are vague and indefinite because "a first channel" appears to lack antecedent basis. Claim 32 has been amended to more clearly provide antecedent support for because "a first channel." Applicants respectfully request that the rejections under 35 U.S.C. § 112 be withdrawn.

C. Rejections under 35 U.S.C. § 102

1. The Church reference

Claims 32-38 have been rejected under 35 U.S.C. § 102(b) as being anticipated by U.S. Pat. No. 5,795,782 by Church et al. (the Church reference). Specifically, the Examiner alleges that the Church reference anticipates the instant claims because the reference discloses a method of detecting an individual polymer molecule using an interface with an ion permeable passage by measuring the ionic conductance of the passage as each monomer interacts with it. Applicants respectfully traverse.

The instant claims are directed to a method of detecting the presence of an analyte in a sample using a pore assembly comprising a number of pore-subunit polypeptides wherein at least one of the pore-subunit polypeptides is a modified pore-subunit polypeptide. The term modified pore-subunit polypeptide is defined in the specification as a pore-subunit polypeptide that is covalently linked to a sensing moiety. *See*, p. 6, ll. 23-27. A modulation in the electrical current through the channel of the pore compared to the current measurement in a control sample lacking the analyte indicates the presence of analyte in the sample.

The Church reference does not anticipate the instant claims because it does not disclose every element of the claimed invention sufficiently so as to allow one of skill in the art to practice the claimed invention. The Church reference is directed to methods that involve measuring conductance measurements across an interface, although not for the purpose of detecting the presence of an analyte, rather for the purpose of characterizing a linear polymer molecule as the linear polymer traverses the interface and the monomers of the polymer interact with the interface. *See*, abstract and FIG. 1 of the Church reference. The Church reference proposes several systems for obtaining such measurements. *See*, col. 7, ll. 10-34.

One method proposed in the Church reference is to record the process of DNA injection or traversal through a channel pore such as the receptor for bacteriophage lambda (LamB) or α-hemolysin that has been isolated on a membrane patch or inserted into a synthetic lipid bilayer. *See*, col. 7, ll. 18-23 and FIG. 1. The Church reference proposes to monitor the conductance of single LamB pores while bacteriophage lambda injects its DNA through the pore. *See*, col. 14, l. 35 - col. 15, l. 4, and col. 16, l. 25 - col. 17, l. 3. FIG.1 of the Church reference depicts an embodiment using α-hemolysin, wherein nucleic acid polymers are threaded through the pore and the monomeric charges and physical obstruction alters the ionic conductance through the pore. *See*, col. 19, l. 2 - col. 20, l. 45.

This embodiment differs from the instant claims because it does not use a modified pore subunit and does not have a sensing moiety covalently linked to a pore sub unit. "For a prior art reference to anticipate in terms of 35 U.S.C. § 102, every element of the claimed invention must be identically shown in a single reference." *In re Bond*, 910 F2.d 831, (Fed. Cir. 1990), (quoting *Diversitech Corp. v. Century Steps, Inc.*, 850 F.2d 675, 677 (Fed. Cir. 1988)). This embodiment of the Church reference does not anticipate the instant claims because at least one element, i.e., a covalently linked sensing moiety, is missing.

Another proposal of the Church reference is to fuse a DNA polymerase molecule to a pore molecule and allow the polymerase to move DNA over the pore's opening while monitoring the conductance across the pore. *See*, col. 7, ll. 24-27 and FIG. 2. The reference suggests that fusion proteins can be constructed from LamB and T7 RNA polymerase wherein both the pore and the polymerase are functional. *See*, col. 7, ll. 15-17. This proposal is clearly prophetic and the Church reference does not demonstrate that such a fusion protein currently exists. For example, the description of FIG. 2 indicates that the figure is a schematic representation of what

such a fusion might look like. *See* col. 5, ll. 63-64. The figure description acknowledges that the drawing of the pore-polymerase combination is simply pieced together from drawings of a polymerase molecule from one literature source and a porin model from another literature source. *See*, col. 6, ll. 10-13. Likewise, the data depicted in FIG. 3 is a not actual data, rather, it is a schematic representation of what such data might look like. *See*, col. 6, ll. 14-15. There is no indication that Church et al. actually posses the proposed sensor. More importantly, they do not provide sufficient disclosure so as to enable one of ordinary skill in the art to make such a device.

Referring to FIG. 2, it is apparent that the proposed fusion protein is so complex that one of ordinary skill in the art would require extensive disclosure in order to make such a construct. Church, et al. propose that LamB and T7 RNA polymerase can be fused to make the combination pore-polymerase device. They propose that such a fusion can be translated from one or more natural and/or recombinant DNA molecule(s) which includes a first DNA which encodes a channel or pore and a second DNA which encodes a monomer-interacting portion of a monomer polymerizing agent. *See*, col. 3, ll. 38-44. However, for one of ordinary skill in the art to actually construct such a natural or recombinant DNA for encoding a functional pore-polymerase combination, they would need to know the experimental mutagenesis details such as preferred strains of bacteria, phage and plasmids, etc. No such disclosure is provided by the Church reference.

Church, et al. assert that any permissive sites in LamB can be targeted for the proposed fusion. *See*, col. 15, ll. 15-20. However, they do not teach where on the LamB protein these permissive cites are located. Instead, they incorporate references by Boyd et al. and by Ehrmann et al. *See*, col. 15, ll. 21-23. These references are not even concerned with fusions of LamB and

T7 RNA polymerase, rather, they are directed to fusions of alkaline phosphatase with the E. coli membrane protein MalF. *See*, Boyd and Ehrmann references, attached as Appendices A and B, respectively. These references do not in any way provide enabling disclosure of how to make the fusion protein proposed in the Church reference. Furthermore, it is impermissible to incorporate essential material, i.e., material relied upon to provide enabling disclosure, from non-patent publications. *See* MPEP 608.01(p).

Church et al., propose that T7 RNA polymerase is best fused to the C-terminal end of LamB because the polymerase is known to function in protein fusions with this orientation. *See*, col. 15, ll. 25-29. However, the only support for this proposition is the incorporation by reference of an article by Ostrander et al. *See*, col. 15, ll. 29-30. (There is evidently a mistake in this citation to Ostrander et al., 1990, *J. Bacteriol.*, 116:1436-46. There does not appear to be a publication by Ostrander in *J. Bacteriol.* in 1990, as cited. *See* the cumulative Author Index for the 1990 volume of *J. Bacteriol*, attached as Appendix C. The 1990 volume of *J. Bacteriol.* is vol. 172, as opposed to vol. 116, and the article beginning on page 1436 of this volume is by Francios et al., not Ostrander et al. See attached Appendix D. Vol. 116 of *J. Bacteriol.* was published in 1973, but the article beginning on page 1436 of this vol. is by Randall-Hazelbauer et al., not Ostrander et al. See attached Appendix E. Neither the Francios article nor the Randall-Hazelbauer article have anything to do with T7 RNA.)

In summary, the only support that the Church reference offers for the proposed pore-polymerase combination is impermissible incorporation by reference of research articles that themselves are not concerned with such a pore-polymerase combination, and one of which is incorrectly cited. This is clearly not enabling support for the invention claimed in the present application.

A reference must describe a claimed invention sufficiently to place the public in possession of the invention if the reference is to qualify as prior art under 35 U.S.C. § 102(b). *In re Donohue*, 766 F.2d 531, 533 (Fed. Cir. 1985). Even if the claimed invention is disclosed in a printed publication, that disclosure will not suffice as prior art if the disclosure is not enabling. *Id.* While it is not necessary that an invention disclosed in a publication have actually been made in order to constitute prior art, it is necessary that the invention be described sufficiently to allow one of ordinary skill in the art to make the invention. *Id.* The Church reference does not describe the presently claimed invention with enabling sufficiency. Applicants therefore respectfully request that the rejection under 35 U.S.C. § 102(b) be withdrawn.

2. The Braha reference

Claims 32-33, 35 and 38 were rejected under 35 U.S.C. 102(e) as being anticipated by Braha et al., *Chemistry & Biology*, 4(7): 497-505, 1997 (the Braha reference). Specifically, the Examiner alleges that the Braha reference anticipates the instant claims because it discloses a method of detecting divalent metal ions using a bacterial pore-forming protein having receptor sites. Applicants respectfully traverse.

The Braha reference is directed to a biosensing architecture utilizing an α-hemolysin, in which pore-subunits have been engineered to contain a binding cite for a divalent metal ion. *See*, Braha reference, abstract and Figure 1. This was accomplished by introducing four histidines into the amino acid sequence of one of the peptides via mutagenesis such that the four histidines would project into the lumen of the pore channel to form a cluster of imidazole sidechains capable of binding to a divalent metal. *See*, Braha reference, p. 499, col. 1, ll. 43-48. The only modifications to the pore-subunit polypeptides disclosed in the Braha reference that are relevant

to the sensing mechanism are mutations within the amino acid sequence of the polypeptide itself, i.e., the peptides comprise only an "endogenous" sensing moiety.

In contrast, the instant claims are directed to a method of detecting an analyte using a pore assembly wherein at least one of the pore-subunit polypeptides is modified to contain a covalently linked to a sensing moiety capable of binding with the analyte. Referring to the instant specification at p. 3, ll. 10-18, the term "modified, pore-subunit polypeptides," according to the instant claims, refers to a pore-subunit polypeptide covalently attached to an "exogenous" sensing moiety. The specification specifically distinguishes the claimed modifications from those taught in the Braha reference, wherein the only modification is one or more mutations within the amino acid sequence of the polypeptide itself. *See*, instant specification at p. 3, ll. 23-25.

The Braha reference does not teach every element of the instant claims because it does not teach a covalently linked sensing moiety. As discussed above, every element of the claimed invention must be identically shown in a single reference if that reference is to qualify as anticipating prior art. Because the Braha reference does not teach every element of the claims, Applicants respectfully request that the rejection under 35 U.S.C. § 102(e) be withdrawn.

The Examiner is invited to contact the undersigned patent agent at 713-787-1558 with any comments relating to the referenced patent application.

Respectfully submitted,

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Date: Sept. 25, 2002

Marked up version of amended claim showing changes made.

32. (twice amended) A method of detecting the presence of an analyte in a sample, the method comprising:

contacting said sample with a pore assembly comprising [a number of] *one or more* pore-subunit polypeptides sufficient to form a pore, *the pore comprising at least a first channel,* wherein at least one of said pore-subunit polypeptides is a modified pore-subunit polypeptide comprising a pore-subunit polypeptide covalently linked to a sensing moiety *capable of binding with the analyte*; and

detecting an electrical current through at least a first channel, wherein a modulation in current compared to a current measurement in a control sample lacking said analyte indicates the presence of said analyte in said sample.

Appendix A

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Genetics

Determinants of membrane protein topology

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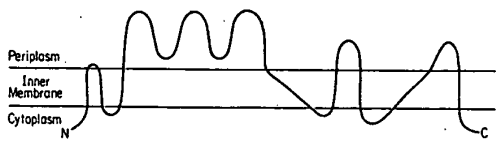
Contributed by Jon Beckwith, August 7, 1987

ABSTRACT The topology of the integral membrane protein MalF, which is required for maltose transport in *Escherichia coli*, has been analyzed using fusions of alkaline phosphatase (EC 3.1.3.1). The properties of such fusion strains support a MalF structure previously proposed on theoretical grounds. Several transmembrane segments within MalF can act as signal sequences in exporting alkaline phosphatase. Other transmembrane sequences, in conjunction with cytoplasmic domains, can stably anchor alkaline phosphatase in the cytoplasm. Our results suggest that features of the amino acid sequence (possibly the positively charged amino acids) of the cytoplasmic domains of membrane proteins are important in anchoring these domains in the cytoplasm. These studies in conjunction with our earlier results show that alkaline phosphatase fusions to membrane proteins can be an important aid in analyzing membrane topology and its determinants.

We recently described a genetic approach to analyzing the topology of integral membrane proteins (1). Using a membrane protein with a relatively simple and well-defined structure (the *tsr*-encoded serine chemoreceptor of *Escherichia coli*), we showed that the properties of alkaline phosphatase (EC 3.1.3.1) fusions to the protein correlated with its topology. Alkaline phosphatase fused to the extracytoplasmic (periplasmic) domain of Tsr protein exhibited high alkaline phosphatase enzymatic activity. Alkaline phosphatase fused to the cytoplasmic domain of the protein showed very low enzymatic activity. This strict correlation of the enzymatic activities of hybrid proteins with the cellular location of the domain to which alkaline phosphatase was fused suggested that this fusion approach could be used to determine the topologies of proteins of unknown disposition in the membrane.

In this paper, we present experiments that extend this approach to a membrane protein of complex topology. This protein, the *malF* gene product, is required for maltose transport in *E. coli* (2). The DNA sequence of the *malF* gene revealed the existence of eight possible transmembrane sequences within the protein (3). These putative transmembrane segments are composed of stretches of 20 or more amino acids that are uncharged and have a high proportion of hydrophobic amino acids.

The amino acid sequence of *MalF* suggested the model (shown below) for the arrangement of the protein in the membrane (3).



The transmembrane segments of MalF protein in the model correspond to the hydrophobic sequences. The amino terminus of MalF was positioned in the cytoplasm because of a cluster of positively charged amino acids that precedes the first presumed transmembrane segment. von Heijne (4) has shown that the charge distribution around transmembrane segments is such that there is usually a net positive charge at the cytoplasmic end of such stretches. The representation of the fourth and seventh transmembrane segments implies nothing about their actual structure but simply reflects the longer lengths of the hydrophobic stretches in these two cases. Support for this structure for MalF comes from studies on fusions of β-galactosidase to the MalF protein (5).

In this paper, we describe experiments that show that fusions of alkaline phosphatase to the MalF protein have properties that are consistent with the proposed structures. These results indicate that the alkaline phosphatase fusion approach can be a useful aid in analyzing the membrane arrangement of proteins of complex as well as simple topologies.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. *E. coli* strains used were CC118(F'*lacI*^{Q} *pro*) (ref. 6); DHB3, which is *araD139* Δ(*ara–leu*)7697 Δ*lacX74* Δ*phoA Pvu* II (ref. 7) *phoR* Δ*malF3* [a nonpolar deletion of the *malF* gene (ref. 3) from base pair −88 to base pair 1404] *galE galK thi rpsL*; DHB4, which is DHB3 (F'*lacI*^{Q} *pro*); DHB24, which is DHB4 *pcnB zad*::Tn*10* (ref. 8); DHB5060, which is *lacZ*$_{am}$ *argE*$_{am}$ *rpoB rpsL araD*139 Δ(*ara–leu*)7697 *galE galK thi hsr*$^-$ *hsm*$^+$ λ CH616 [which expresses M13 gene II (ref. 9)] pOXgen [a derivative of the F factor pOX38 (ref. 10) carrying a gene for resistance to gentamycin (ref. 11)] pIQ [a derivative of pACYC184 (ref. 12) carrying *lacI*^{Q}]; and BW10724, which is (λ*recA*$^+$) *recA*::*cat* Δ*lac*U169 *pho-510 thi rpsL*, a gift of B. Wanner. The plasmid pSF691 is described in ref. 3; pHS17 (a gift of H. Shuman) contains the *E. coli malF* gene and part of the *malG* gene expressed under the *tac* promoter of pKK223–3 (ref. 13); and pDHB32 is similar but contains the M13 intergenic region from pZ150 (ref. 14).

Media and Enzymes. Media were made according to Miller (15). T4 DNA ligase was purchased from Pharmacia. T4 DNA polymerase and T4 gene *32* protein were obtained from Boehringer Mannheim. All other enzymes were obtained from New England BioLabs and were used according to the manufacturer's suggestions.

Assay of Alkaline Phosphatase. Fresh stationary-phase cultures in LB broth were diluted 100-fold and were grown for 2 hr and 40 min at 37°C before assay as described (16). A background of 0.1 unit has been subtracted from each value.

Isolation of Transposon Insertions. Transposon insertions were isolated using two different protocols. The F*lac*–Tn*phoA* method (6) was used to isolate fusions d2b and d4a in pSF691/CC118(F'*lacI*^{Q} *pro*), fusions 17-1 and 17-3 in pHS17/CC118(F'*lacI*^{Q} *pro*), and fusions 160, 121, 165, and 3-826 in pDHB32/DHB4. Fusions 4.30c, 4.39b, and 4.29c in

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pDHB32/DHB24 were isolated using λ Tn*phoA* as described (17), except that 5×10^8 phage particles were used to infect 2×10^9 cells in 5 ml of LB broth. After 1 hr at 37°C, the culture was diluted into 100 ml of LB broth containing kanamycin (30 μg/ml) and ampicillin (100 μg/ml) and was divided into 50 aliquots, which were incubated overnight at 37°C. Phage f1IR1 (18) lysates were prepared from 0.1 ml aliquots of each of the 50 independent, resistant cultures as described (15). These lysates have approximately equal numbers of phage particles and transducing particles that contain single-stranded plasmid DNA. The lysates were used to transduce DHB24 with selection for resistance to kanamycin (40 μg/ml) and ampicillin (200 μg/ml) to isolate transductant cells carrying plasmids with transposon insertions. Between 0.1% and 10% of the transductant colonies were blue on plates containing the chromogenic indicator of alkaline phosphatase activity, 5-bromo-4-chloro-3-indolyl phosphate. Prior to analysis, fusions not isolated in pDHB32 were cloned into this plasmid *in vitro*.

DNA Sequence Analysis. Plasmids coding for fusion proteins were reduced in size by deletion of the transposase and kanamycin resistance genes *in vitro* using *Sal* I and *Xho* I restriction endonucleases (Fig. 1). Dideoxy sequence analysis (19) to locate function positions was carried out using single-stranded template DNA obtained from f1IR1 phage lysates grown on plasmid-containing strains (14).

Oligonucleotide-Directed Deletion Mutagenesis. To construct *phoA* fusions to selected sites in *malF* such that the fusions are isogenic to those generated by Tn*phoA* transposition, we synthesized 43- to 48-mer oligonucleotides with 23 residues complementary to the 5′ end of Tn*phoA* and the rest complementary to the target site in *malF*. [The oligonucleotides were prepared by Steve Lin on Applied Biosystems (Foster City, CA) apparatus.] Oligonucleotides were phosphorylated with T4 polynucleotide kinase, and 40 ng was annealed to 2 μg of single-stranded DNA prepared from an f1IR1 phage lysate grown on a strain containing the parent fusion plasmid (Fig. 1). The oligonucleotide primer was extended by incubation for 2 hr at 37°C with T4 DNA polymerase, T4 gene *32* protein, and T4 DNA ligase (20). DNA remaining single stranded was digested with mung bean nuclease. Between 1% and 50% of the transformants obtained in DHB24 contained plasmids with the new fusion.

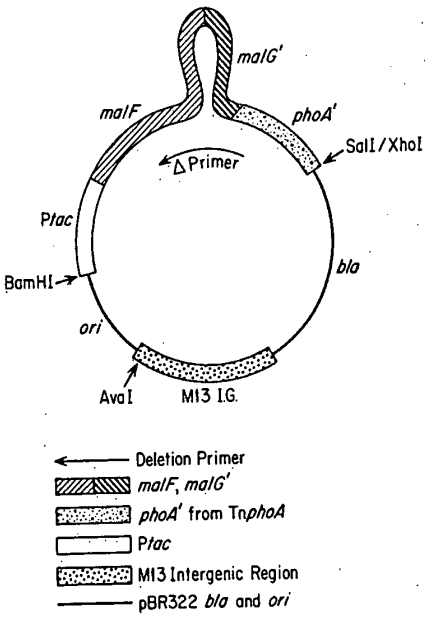

FIG. 1. Oligonucleotide-directed deletion mutagenesis and insertion of fusions onto the chromosome. A precise deletion was generated in a parental *malG–phoA* fusion plasmid by elongating a deletion primer using a single-stranded plasmid DNA template prior to digestion of the single-stranded loop with a single-strand specific endonuclease followed by transformation. For further analysis of each fusion thus generated, the plasmid replication origin, *ori*, was deleted from plasmid DNA using the *Bam*HI and *Ava* I sites shown. This generates a plasmid that uses the M13 phage origin for autonomous replication and must insert into the chromosome to transform stably a host not expressing M13 gene II protein. The plasmid shown has the *tac* promoter (P*tac*) (13), *malF*, a Tn*phoA* fusion to *malG*, the *Sal* I/*Xho* I site where most of the Tn*phoA* DNA has been deleted, the *bla* gene of pBR322, the M13 intergenic region (I.G.) (14), and the pBR322 replicative origin.

Recombination of Fusions into the Chromosome. A derivative of each of the pDHB32 fusion plasmids was constructed in which the plasmid replicative origin was deleted using restriction endonucleases *Bam*HI and *Ava* I as shown in Fig. 1. DNA molecules that contain the M13 intergenic region but have no plasmid replicative origin can be maintained as plasmids in strains (such as DHB5060) that express the M13 gene II protein (9). Origin deletion plasmid DNA containing each fusion was prepared from a DHB5060 derivative and used to transform DHB4 by selecting resistance to ampicillin (25 μg/ml). The transformants, which were obtained at a frequency about 10^{-6} of that expected for origin-containing plasmid DNA, appear to have one or more copies of the originless plasmid integrated by recombination at the *phoA* locus on the chromosome. Integrated plasmids were stabilized by transduction of *recA*::*cat* into the strains. The number of integrated plasmids in some of these strains was determined to be only one by testing f1IR1 lysates prepared on them for transducing particles. Strains with two or more originless plasmids integrated at *phoA* package transducing particles efficiently, whereas those with only one originless plasmid package fewer (by a factor of 10^{-6}) transducing particles (unpublished results).

Proteolysis and Fractionation. Osmotic shock fractionation was carried out as described (1) except for the differences noted below. Exponential cultures were induced with 5 mM isopropyl-β-D-thiogalactoside (Bachem, Torrance, CA) for 30 min before washing. They were then pulse labeled with 60 μCi of [^{35}S]methionine per ml (10^6 Ci/mol; Amersham; 1 Ci = 37 GBq) for 1 min and were incubated in the presence of unlabeled methionine for 10 min. Washed cells were resuspended in spheroplast buffer (0.1 M Tris·HCl, pH 8/0.5 M sucrose/0.5 mM EDTA), incubated 5 min on ice, and centrifuged. The pellet was warmed to room temperature, resuspended in ice-cold water with or without trypsin (type XIII, Sigma), incubated for 5 min on ice, and separated into periplasmic and cellular (membranes plus cytoplasm) fractions by centrifugation in the presence of 0.5 mM phenylmethylsulfonyl fluoride (Sigma). The two fractions were then precipitated with trichloroacetic acid and were analyzed by immunoprecipitation and sodium dodecylsulfate polyacrylamide gel elecrophoresis ($NaDodSO_4$/PAGE) (6). Parallel unlabeled preparations were assayed to determine the fractionation of alkaline phosphatase activity (16) and a cytoplasmic marker, glucose-6-phosphatase dehydrogenase activity (21).

RESULTS

***In Vivo* Fusions and Properties.** We have isolated *malF–phoA* gene fusions after transposition of the transposon Tn*phoA* into plasmids carrying *malF* (see *Materials and Methods*). Transposition events resulting in significant alkaline phosphatase activity were identified by the formation of pale blue to dark blue colonies on media containing the

indicator dye 5-chloro-3-bromo-indolyl phosphate. To determine the sites of fusion of alkaline phosphatase to MalF, plasmids were analyzed by restriction mapping and by DNA sequencing. In addition, levels of alkaline phosphatase activity in permeabilized cells carrying the fusion plasmid were assayed. Finally, the amounts and stabilities of the hybrid proteins were analyzed by precipitation with anti-alkaline phosphatase antibody and $NaDodSO_4$/PAGE.

The results of these studies are summarized in Fig. 2. Fusions at nine different sites in MalF had similarly high levels of alkaline phosphatase activity. This group comprised fusions in each of the four proposed periplasmic domains of MalF. These fusion strains all made stable fusion proteins of the predicted size (data not shown). Two other fusion plasmids isolated from transformant colonies showing paler blue color had fusion joints at the carboxyl end of a membrane-spanning stretch crossing from the periplasm to the cytoplasm according to our model for MalF (fusions 17-1 and d2b, see Fig. 2). These fusions are at sites predicted to be cytoplasmic in the model, yet they show 30–100% of the enzymatic activity of fusions to the periplasmic domains. In our previous studies with a membrane protein of simpler structure, alkaline phosphatase fusions to the cytoplasmic domain exhibited enzymatic activities only 2–5% of those seen with fusions to the periplasmic domain (1). We obtained no other fusions to putative cytoplasmic domains by this approach.

It seemed possible that the intermediate activity of fusions 17-1 and d2b was due to the absence of amino acid sequences more carboxyl terminal to the fusion joints in the corresponding hybrid proteins. The missing sequences, which include the entire putative cytoplasmic domain following this transmembrane segment, could be important in anchoring this part of the protein in the cytoplasm. In this case, fusions to other portions of cytoplasmic domains might have alkaline phosphatase more stably localized to the cytoplasm and would show lower activity. These might be found among the white or very pale blue colonies in transposition selections. Nine independent colonies of these latter types were analyzed. In all cases, DNA sequencing of the hybrid genes showed that they were the result of out-of-frame Tn*phoA* insertions rather than in-frame fusions giving low alkaline phosphatase activity. Given these findings, searching for the predicted low activity additional cytoplasmic domain fusions using this approach appeared impractical. Therefore, to test our explanation for the intermediate activity of fusions 17-1 and d2b, we made use of an *in vitro* technique to generate additional fusions at critical positions in the presumptive MalF cytoplasmic domains.

In Vitro Fusions. Our further analysis of the model for MalF protein is designed to test whether cytoplasmic loops between membrane-spanning stretches of integral membrane proteins contain information that contributes to the topological arrangement of the proteins. We constructed fusions to each of the MalF protein cytoplasmic loops, which differ only in whether they are positioned at the very beginning or very end of the loop. If sequences within the loop contribute to the loop's stable cytoplasmic localization, fusions at the ends of the loops in which the loop is present should show less activity than those at the beginning of the loops in which the loop is deleted.

We have used oligonucleotide-directed deletion mutagenesis to construct the set of fusions. This was done in the following way (see Fig. 1). We began with either a late *malF–phoA* fusion or a *malG–phoA* fusion (unpublished results). (The *malG* gene is distal to *malF* in an operon, and part of it is present in the plasmid used.) Oligonucleotides were synthesized that contained 23 nucleotides of the end of Tn*phoA* that generates the fusion and 20–25 nucleotides of the target sequence in *malF*. These oligonucleotides were used to prime second-strand synthesis with a single-stranded parental template. Such fusions are isogenic to those obtained by transposition of Tn*phoA* in the sense that they substitute exactly the same fusion joint sequence at different points in *malF*. This technique was used to generate fusions 0-55, II-175, II-205, IV-958, VI-1177, VI-1249, and VIII-1548 (see Fig. 2).

We determined the alkaline phosphatase activity of these fusions. Because the particular plasmid we used was unstable and this instability interfered with accurate comparison of the activity of different fusions, we recombined all of the in-frame fusions onto the chromosome. The results of assays of these fusions are summarized in Fig. 2. They show the following: (*i*) Fusions to periplasmic domains all show 20–30 units of alkaline phosphatase activity. (*ii*) Fusions to cytoplasmic domains show up to 200 times lower activity, but they vary greatly. (*iii*) Fusions to the carboxyl ends of cytoplasmic loops have 7–20 times lower activity than fusions to the amino-terminal ends of the same loops. (*iv*) The fusions following the second membrane-spanning stretch have at least ten times lower levels of activity than the corresponding fusions following the fourth such stretch, whereas the fusions following the sixth stretch are intermediate. (*v*) The fusion 0-55, which deletes all hydrophobic regions of MalF, has no detectable alkaline phosphatase activity. This fusion protein is stable (data not shown). (*vi*) The VIII-1543 fusion, which

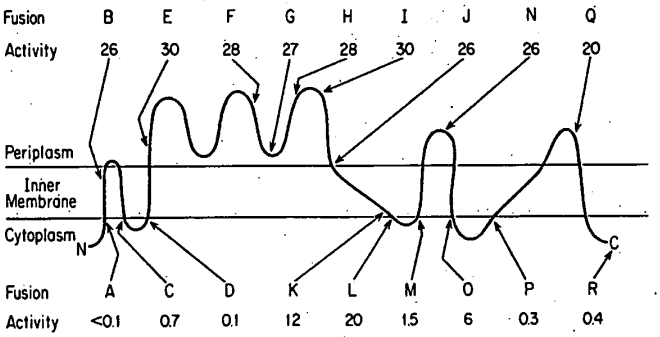

FIG. 2. Alkaline phosphatase activity of *malF–phoA* fusion strains. The alkaline phosphatase activity of each *malF–phoA* fusion is shown with an arrow pointing to the site at which the fusion joint is located in the topological model of *malF*. Assays were carried out using strains with a single copy of the fusion stably integrated into the chromosomes. At least two independent insertions of each fusion were assayed, and the values shown are averages. A is fusion 0-55, fused at base 55; B, 17-3 at 94; C, II-175 at 175; D, II-205 at 205; E, 160 at 295; F, 4.30c at 607; G, 121 at 649; H, 165 at 682; I, d4a at 712; J, 3-826 at 826; K, 17-1 at 907; L, d2b at 920; M, IV-958 at 958; M, 4.39b at 1051; O, VI-1177 at 1177; P, VI-1249 at 1249; Q, 4.29c at 1417; R, VIII-1548 at 1548.

has alkaline phosphatase fused to the carboxyl-terminal amino acid of MalF has a MalF$^+$ phenotype, whereas all other fusions are MalF$^-$.

Proteolysis and Fractionation. The similar alkaline phosphatase activities of different fusions to presumed periplasmic domains of MalF suggest that the active moieties of these fusion proteins have the same specific activity and that they are positioned in the periplasm, presumably on the periplasmic face of the inner membrane. To test this supposition, labeled cells of one fusion strain were subjected to osmotic shock in the presence of trypsin. This treatment releases the periplasm and makes the outer face of the cytoplasmic membrane accessible to trypsin. Wild-type alkaline phosphatase activity is resistant to trypsin, although the protein is cleaved at a site near the amino terminus (22). Mild trypsin treatment released 86% of the alkaline phosphatase activity of fusion 3-826 from shocked cells. At the same time, less than 15% of the activity of glucose-6-phosphate dehydrogenase, a cytoplasmic protein, was lost from the cells. Analysis of the labeled protein by immunoprecipitation with anti-alkaline phosphatase antiserum and NaDodSO$_4$/PAGE shows a prominent fusion-protein-sized band in the cellular (cytoplasm plus membrane) fraction from untreated cells (Fig. 3). This band is also present in cells treated with trypsin in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (not shown). This band is missing from the cellular fraction of cells treated with trypsin, which instead shows a prominent alkaline-phosphatase-sized band in the periplasmic fraction. β-Galactosidase was also identified by immunoprecipitation and recovered in the cellular fractions of both treated and untreated cultures (Fig. 3). These results indicate that the alkaline moiety of the fusion protein is predominantly localized at the outer face of the inner membrane.

DISCUSSION

In this paper we have extended the alkaline phosphatase fusion approach for analyzing membrane protein topology to a complex membrane protein, MalF. The model for MalF topology proposed in Fig. 2 was originally based on the amino acid sequence of the protein (3). The properties of β-galactosidase fusions to MalF are consistent with this model (5). Properties of alkaline phosphatase fusions to MalF follow a relatively simple pattern, which is also consistent with the proposed structure. All fusions to proposed periplasmic domains show high alkaline phosphatase activity with about the same activity for different fusions. In contrast, fusions to proposed cytoplasmic domains show activities that are slightly lower to 200 times lower than those of the periplasmic domain fusions.

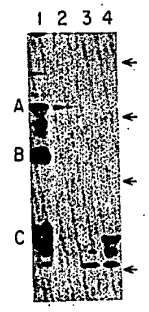

FIG. 3. Proteolysis and fractionation of an active MalF–PhoA fusion protein. Cells producing the 3-826 fusion protein were fractionated after incubation in the presence or absence of trypsin. Proteins precipitated by a mixture of antibodies to alkaline phosphatase and β-galactosidase were separated by NaDodSO$_4$/PAGE. Lanes: 1 and 2, immunoprecipitate from the cellular fraction (membrane plus cytoplasm); 3 and 4, immunoprecipitate from periplasmic fraction. Lanes 1 and 3 have been subjected to osmotic shock in the absence of trypsin. In lanes 2 and 4 the osmotic shock was carried out in the presence of trypsin (5 μg/ml) at 0°C for 5 min. The molecular weights of markers (prestained; Bethesda Research Laboratories) indicated by the arrows at right are from top to bottom: 200, 97.4, 68, and 43 kDa. Band A is β-galactosidase, B is the fusion protein 3-826, and C is alkaline-phosphatase-sized.

The high activity of all fusions in which alkaline phosphatase is attached to a presumed periplasmic domain of malF indicates that each of the transmembrane segments preceding these domains is competent to initiate export of alkaline phosphatase. In this regard, these sequences act like the cleavable signal sequences of secretory proteins. Similar suggestions have been made for other integral membrane proteins (23–25). Proteolysis and cell fractionation studies of one fusion strain in this class, 3-826, confirm the assumption that high enzymatic activity of the hybrid correlates with efficient export of the alkaline phosphatase moiety. We presume that the transmembrane sequences promoting export of alkaline phosphatase in the fusion proteins play a similar role in determining the topological structure of the native protein.

The alkaline phosphatase activity of a fusion to a MalF protein cytoplasmic domain depends on the position of the fusion joint within that domain. Fusions of alkaline phosphatase to the very amino terminus of a cytoplasmic domain (i.e., in which none of that cytoplasmic domain is included in the hybrid protein) show 7- to 20-fold higher activity than those more carboxyl terminal in the same cytoplasmic domain. This pattern suggests that cytoplasmic domain sequences help to anchor alkaline phosphatase in the cytoplasm in such hybrid proteins. In other words, the hydrophobic transmembrane segment itself is not the sole determinant of stable anchoring. We presume that these same cytoplasmic sequences that stabilize alkaline phosphatase in the cytoplasm in such hybrids contribute to determining the topology of the unfused MalF protein in an analogous way. We suspect that positively charged amino acid residues near the cytoplasmic ends of membrane-spanning stretches may play a primary role in stabilizing such stretches. An analysis of many integral membrane proteins by von Heijne (4) suggests that these charged residues are a common structural feature of such domains and that they may play a role in determining the orientation of the membrane-spanning segments.

Other features of the determinants of membrane protein topology are suggested by a comparison of fusions in which alkaline phosphatase is fused to cytoplasmic domains of MalF. In particular, the fusions to the cytoplasmic domain following the fourth membrane-spanning segment have 10- to 20-fold higher activities than the corresponding fusions following the second such sequence. We can imagine various factors that might contribute to this dramatic difference. First, it is possible that properties of individual membrane-spanning segments or the hydrophilic domains themselves determine the efficiency with which alkaline phosphatase is retained in the cytoplasm of the fusion strains. For instance, the fourth hydrophobic sequence is quite long (32 amino acids) compared to others oriented in the same direction. Conceivably, it is long enough so that an amino-terminal portion of it could span the membrane and a carboxyl-terminal portion act as a "signal sequence" in the cytoplasm allowing efficient export of alkaline phosphatase. Alternatively, the large periplasmic hydrophilic domain that precedes the fourth membrane-spanning segment may destabilize the membrane interactions at its carboxyl terminus. In both of these cases, it seems unlikely to us that these sequences would behave this way in an intact MalF protein

but rather function aberrantly in the absence of the remaining carboxyl terminus of the protein.

Second, it may be that interactions between domains are normally important in determining the topology of the protein. For example, close packing in the membrane of the first two transmembrane stretches could contribute to the stabilization of the fused alkaline phosphatase in the cytoplasm, whereas the fourth transmembrane stretch may have no such interactions available to it in the relevant fusion proteins. We expect that these alternatives will be distinguishable by the engineering of further derivatives of the fusion strains.

The alkaline phosphatase moiety of hybrid protein 3-826, a high activity fusion, is fully exported to the periplasm (Fig. 3). Proteolysis and fractionation experiments with fusions having intermediate activity (such as 17-1) to determine the disposition of their alkaline phosphatase moieties have not been done. Two explanations for the alkaline phosphatase activity of these latter fusions are possible. The proteins may be efficiently localized to one compartment, periplasm or cytoplasm, and may have a specific activity lower than fusions such as 3-826. Alternatively, they may be partially exported and have a periplasmic fraction of high specific activity and a cytoplasmic fraction of low specific activity. Experiments with alkaline phosphatase signal sequence mutants (7, 26) suggest that the second alternative is likely to be correct. Cytoplasmic alkaline phosphatase is inactive and unstable. Stabilization of cytoplasmic alkaline phosphatase does not lead to increased activity or export. This finding suggests that cytoplasmic alkaline phosphatase may enter a nonexportable pool. The relative rates of export and entry into this pool may determine the level of activity of fusions with intermediate activity.

Our results suggest that isolating Tn*phoA* fusions by transposition alone may not provide a complete analysis of complex membrane proteins that have small, but critical, cytoplasmic domains. Our *in vitro* oligonucleotide deletion mutagenesis approach may be a necessary adjunct to *in vivo* methods for studying such proteins. The properties of fusions generated *in vitro* can be directly compared to those generated by Tn*phoA* transposition because the fusion joint segments are the same. As a general experimental approach, we believe that initial results with Tn*phoA* fusions generated by transposition will allow construction of a working model, which can be tested with specific fusions at critical points generated by the *in vitro* method.

To use the oligonucleotide deletion method, one needs to begin with either a very late fusion or a fusion to a distal gene obtained *in vivo*. By using 40- to 50-mer oligonucleotides with one part complementary to the end of Tn*phoA* and the other complementary to the target sequence, it is possible to recover the desired new fusions with high efficiency.

Fusions of the type described here replace the carboxyl-terminal part of the protein being studied with the probe alkaline phosphatase. The properties of such fusions are thus determined solely by the amino-terminal portion of the protein. In cases in which the arrangement of a membrane protein is determined by interactions between its amino-terminal and carboxyl-terminal portions, a fusion analysis may not give a valid picture of the final topology. In the case of the MalF protein, the finding that the longest *malF–phoA* fusion retains MalF activity indicates that the MalF protein moiety has assumed its proper conformation in the membrane. The low alkaline phosphatase activity of this fusion indicates that the carboxyl-terminal domain of MalF is normally cytoplasmic as was originally predicted from the orientation of the rest of the protein. The results suggest, then, that alkaline phosphatase fusions are not, in general, disrupting the orientation of segments of the protein, since we obtain an internally consistent picture from this analysis.

The consistency of the results presented here and their coherence with results obtained from *lacZ* fusions to *malF*, in addition to our earlier results with a simpler membrane protein, indicate that the alkaline phosphatase fusion approach can be generally used to analyze membrane protein topology.

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Genetics

Genetic analysis of membrane protein topology by a sandwich gene fusion approach

(alkaline phosphatase/MalF protein/*phoA* cassette)

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Contributed by Jonathan Beckwith, July 13, 1990

ABSTRACT **We describe a cloning vector that allows the construction of *phoA* sandwich fusions in which mature alkaline phosphatase is inserted into target proteins. In contrast to previous fusions obtained using the Tn*phoA* transposon, the entire amino acid sequence of the target protein is present in the fusion product. We have constructed a series of sandwich fusions of alkaline phosphatase to the multispanning cytoplasmic membrane protein MalF. Despite the fact that the alkaline phosphatase was tethered to MalF at both its N and its C terminus, the enzyme exhibited high activity when it was fused to a periplasmic domain of the membrane protein. Cells harboring an alkaline phosphatase sandwich fusion to the end of the first membrane-spanning segment of MalF exhibited both MalF and alkaline phosphatase activity. When alkaline phosphatase was inserted into a cytoplasmic domain of MalF, its specific activity was very low. Our results suggest that the alkaline phosphatase activity of *phoA* sandwich fusions provides a more sensitive monitor than previous methods of the cellular localization of the domain of the target protein to which the enzyme is fused. Thus, the sandwich fusion approach can give a more accurate picture of membrane protein topology.**

A previous paper (1) described a genetic strategy for analyzing the topology of integral membrane proteins. The approach involves the use of fusions to the *phoA* gene, which codes for alkaline phosphatase (AP) of *Escherichia coli*. The method depends on four features of AP. (*i*) The enzyme is only active when it is translocated through the cytoplasmic membrane into the periplasm. It is inactive when localized to the cytoplasm. (*ii*) The signal sequence of AP can be replaced by export signals derived from other proteins, including those found in integral membrane proteins. (*iii*) The mature part of AP is neutral with respect to export. It contains no export information itself. (*iv*) Extensive amino acid sequences from other proteins can be fused to the N terminus of AP without interfering with its activity. Thus, with a cytoplasmic membrane protein, only AP fusions to periplasmic domains of the protein, which are preceded by an export signal, show high AP enzymatic activity. Fusions to cytoplasmic domains exhibit low activity. As a result, by determining the AP activity of fusions to different hydrophilic domains of such proteins, it is possible to distinguish cytoplasmic from periplasmic domains and, in this way, determine the membrane topology of the protein.

Both *in vivo* and *in vitro* systems for generating *phoA* fusions have been developed. Tn*phoA*, a derivative of Tn*5*, allows the *in vivo* isolation of *phoA* fusions to plasmid-encoded as well as chromosomal genes (2, 3). *In vitro*-constructed fusions can be obtained by the use of plasmids containing the *phoA* gene with conveniently placed restriction sites (4, 5).

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The AP fusion approach has been successfully used to study the topology of a number of cytoplasmic membrane proteins (6). However, we have noted a potential limitation to the approach. The fusions are generated in such a way that AP replaces a C-terminal portion of the membrane protein. If assembly into the correct topological structure depends upon interactions between N-terminal and C-terminal portions of the membrane protein, the fusion approach could give an incorrect picture. While this potential limitation does not appear to have been a problem in most of the analyses done so far, we have found situations in which C-terminal information is required for proper assembly (7, 8). In the case of two cytoplasmic membrane proteins, MalF and leader peptidase, we have found that *phoA* fusions to the beginning (N-terminal) of cytoplasmic domains of the proteins exhibit 7- to 20-fold higher AP activity than fusions to the end (C-terminal) of the same domains. In an extreme case, a fusion to a cytoplasmic domain of the MalF protein exhibited almost as much AP activity as a fusion to a periplasmic domain. In these cases, the AP enzymatic activity corresponds to AP exported to the periplasm (C. A. Lee, D.B., and J.B., unpublished results).

We have shown (9) that the C-terminal information, which is missing in certain fusion proteins and which is required for proper topological assembly of the MalF fusion protein, involves the positively charged amino acids usually found (10) in cytoplasmic domains of such proteins. Positively charged amino acids appear to be required to stably anchor the fused AP as well as the hydrophilic segment of MalF in the cytoplasm.

In this study, in order to overcome the limitation to the Tn*phoA* fusion approach described above, we have constructed a new class of AP fusions. In these fusions, rather than replacing the C-terminus of the membrane protein MalF with AP, we constructed fusions in which AP is inserted into an otherwise intact MalF protein. This was done either by a series of steps using the AP fusions obtained before or by the use of a newly constructed cassette that allows the direct generation of what we call sandwich fusions. In a periplasmic sandwich fusion, AP exhibits normal enzymatic activity even though it is tethered at both its N and C termini. In contrast, cytoplasmic Tn*phoA* fusions that resulted in high levels of AP export, when converted into sandwich fusions, show very low levels of AP activity. The sandwich fusion approach thus provides a more accurate way of examining membrane protein topology as well as providing a tool for the analysis of a number of other biological problems.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. *E. coli* strains used were ME 1201, which is a *recA*::*cat* (taken from strain BW 10724, which was a gift of Barry Wanner, Purdue University)

Abbreviation: AP, alkaline phosphatase.

derivative of DHB4 [*araD139* Δ(*ara–leu*)*7697 lacX74 phoA* Δ[*Pvu* II]*phoR malF3 galE galK thi rpsL* F'*lacI*^{Q} *pro*; ref. 7] and DHB24, which is DHB4 *pcnB zad*::Tn*10* (11). Phage f1R408 (12) was used to prepare single-stranded DNA template from plasmids, which were all derivatives of pDHB32 (7). Phage m13tg130 (13) was used to construct the polylinker at the 3' end of *phoA* in pSWFII. pDHB32 contains the *E. coli malF* gene and part of the *malG* gene expressed under the *tac* promoter of pKK223-3 (14). It also contains the M13 intergenic region (15).

Media, Cell Growth, and Enzymes. Media are according to Miller (16). Proteins were labeled in cultures growing exponentially at 37°C in M63/0.2% glycerol, supplemented with each common amino acid except methionine, by exposing them to [^{35}S]methionine at 50 μCi/ml (1 μC = 37 kBq) for 2 min. T4 gene *32* protein was obtained from Boehringer Mannheim. All the other enzymes were obtained from New England Biolabs.

AP Assay and Maltose Transport Assay. AP activity in strains was assayed by measuring the rate of *p*-nitrophenyl phosphate hydrolysis in permeabilized cells (17). Data for multicopy plasmids were obtained from uninduced logarithmic-phase cultures. Single-copy data were obtained as described (7), after 160 min of induction of log-phase cultures with 5 mM isopropyl β-D-thiogalactoside. The initial rate of maltose uptake was determined as described by Brass *et al.* (18).

Antibody Precipitation and Gel Electrophoresis. These were done as described by Ito *et al.* (19) and Laemmli (20), respectively.

Oligonucleotide-Directed Mutagenesis. This was done as described (7) except that DNA remaining single-stranded was not digested with mung bean nuclease. Where mentioned in the text, transformants were screened for the altered genotype by colony hybridization using the ^{32}P-end-labeled oligonucleotide as a probe (21).

Construction of Sandwich Fusions *in Vitro*. To construct the *malF–phoA* sandwich fusion at the *Pvu* II site, the *Sal* I–*Bst*EII fragment containing *phoA* of pSWFII was filled in with Klenow DNA polymerase and cloned into the *Pvu* II site of *malF*. To construct the malF–phoA sandwich fusion at the *Bss*HII site, the *Xma* I–*Bst*EII *phoA* fragment of pSWFII was filled in with Klenow DNA polymerase and cloned into the *Bss*HII site of *malF* that had been cut back with mung bean nuclease. Both fusions do have two additional amino acids at the C terminus of AP: glycine and tyrosine. At the N terminus of AP the *Pvu* II sandwich fusion has Ser-Thr-Leu-Glu-Asp-Pro-Arg-Val-Pro-Asp added to the 5' Tn*phoA* linker region (2). In the *Bss*HII sandwich fusion, one alanine of MalF was deleted due to cutting back the *Bss*HII site. At the N terminus of AP, Arg-Val-Pro-Asp was added to the 5' Tn*phoA* linker region.

Conversion of *malF*–Tn*phoA* Fusions to *malF'–phoA–'malF* Sandwich Fusions. This set of sandwich fusions was constructed in three steps (Fig. 1). A 56-mer oligonucleotide was introduced into each fusion strain (Fig. 1*A*) in order to remove the stop codon in *phoA* and to extend the reading frame into a polylinker containing the three restriction sites *Bst*EII, *Bss*HII, and *Sac* I which are present in *malF* as unique sites (Fig. 1*B*). In each case this alteration had no effect on phenotype. The desired candidates were identified by colony hybridization using the oligonucleotide as a probe and subsequently tested for the presence of the additional restriction sites. From these constructs a *Bst*EII fragment was isolated that contained *malF* material 5' to *phoA* and *phoA* itself lacking its stop codon (Fig. 1*B*). In the second step, this *Bst*EII fragment was cloned in frame into the *Bst*EII sit of wild-typ *malF* (Fig. 1*C*). At this stage, a rather complex construct had been generated: *malF'–phoA–'malF'–'malF* (Fig. 1*D*). The size of the 'malF' material that had been duplicated during the cloning step was dependent on the fusion joint. It was only 12 base pairs (bp) in the case of the B fusion, but was 824 and 835 bp for the K and L fusions, respectively. The M fusion was converted to a sandwich fusion somewhat differently. Here, instead of using the *Bst*EII site for subcloning of the altered *malF–phoA* fragment, a *Sac* I fragment was used. Thus the duplicat d *'malF'* material was only 354 bp in size. To fuse the 3' end of *phoA* to the fusion joint in *malF* located downstream of *phoA*, the duplicated *'malF'* material was deleted by using specific oligonucleotides for each fusion.

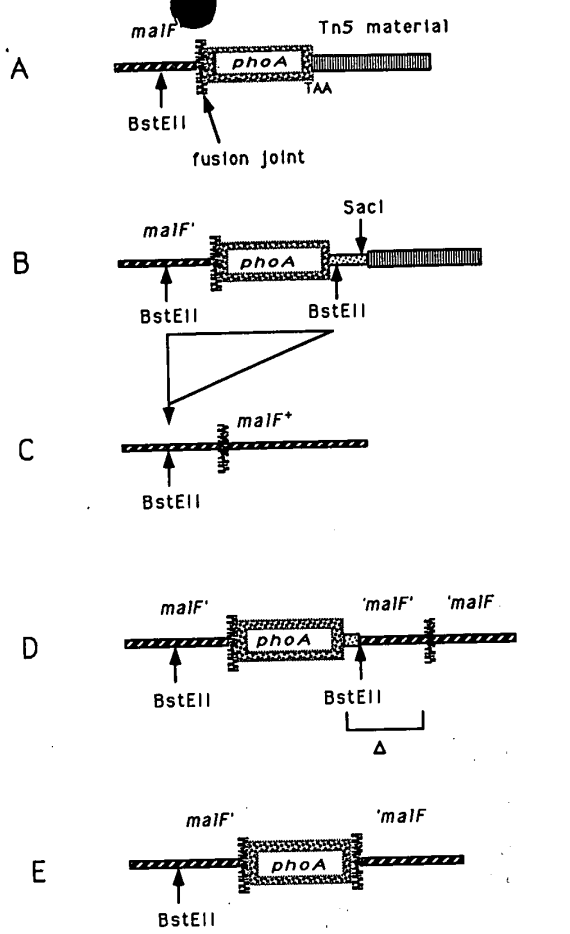


FIG. 1. Conversion of *malF*–Tn*phoA* fusions to *malF'–phoA–'malF* sandwich fusions *in vitro*. (*A*) *malF*–Tn*phoA* fusion. TAA is the termination codon. (*B*) An oligonucleotide is introduced that deletes the stop codon of *phoA* and inserts a linker (stippled box) containing restriction sites. (*C*) The *Bst*EII fragment is excised and cloned into the *Bst*EII site of wild-type *malF*. (*D*) The 3' end of *phoA* is fused to the original downstream fusion joint by deleting the part of *malF* that had been duplicated during the previous cloning step. (*E*) The *malF'–phoA–'malF* sandwich fusion.

Insertion of Sandwich Fusions into the Chromosome in Single Copy. Sandwich fusions cannot be inserted into the chromosome by homologous recombination at the *phoA* locus as *malF*–Tn*phoA* fusions have been (7). Therefore, we have constructed a λ shuttle vector, λDBK261, which permits transfer of fusion genes between plasmids and bacteriophage λ by homologous recombination. This phage contains the Tn*903* kanamycin-resistance gene flanked on one side by the *tac* promoter of DHB32 (7) and on the other side by part of the *bla* gene and part of the M13 intergenic region from the same plasmid. Lysates of DBK261 grown on strains containing pDHB32 derivatives contain recombinant ampicillin-resistant transducing phage in which the missing part of the

bla gene and whatever is between it and the *tac* promoter have replaced the kanamycin-resistance gene. Ampicillin-resistant, kanamycin-sensitive lysogens derived from such phage containing *malF*–Tn*phoA* fusions have AP activities similar to th se reported previously (7). In some cases the identity of the λ recombinants was confirmed by recombination back onto the plasmid shuttle vector pDBK6, which is similar to the phage.

RESULTS

Construction of a *phoA* Sandwich Fusion Cassette. We have constructed a plasmid (pSWFII, Fig. 2) that contains a *phoA* cassette that can be inserted by a simple cloning step at restriction sites in structural genes for proteins. With the appropriate choice of restriction enzymes, the resulting fusion will encode a hybrid protein with AP tethered at both its N and C termini to the target protein. All the sequences from the native target protein are present in the hybrid protein. The plasmid contains most of *phoA* but lacks the coding region for its signal sequenc and five additional amino acids. The *phoA* gene is flanked by polylinkers containing multiple restriction sites. The open reading frame of *phoA* extends into both linker fragments. The polylinker at the 5′ end of *phoA* was taken from pPHO7 (5), a plasmid used for the construction of Tn*phoA* fusions *in vitro*. The polylinker at the 3′ end of *phoA* has a TAG stop codon in the *Xba* I site. It is therefore possible to construct *phoA* sandwich fusions as well as Tn*phoA* fusions by using the same cassette. If the restriction sites upstream of the stop codon are used, sandwich fusions are generated. If the sites downstream of the stop codon are used, fusions comparable to those obtained with Tn*phoA* are generated. The difference from the original Tn*phoA* fusions is the presence of five additional amino acids at the C terminus of AP. These Tn*phoA*-like fusions could be converted to sandwich fusions in a strain carrying an amber suppressor.

pSWFII was used to construct one *malF*–Tn*phoA* fusion and two *malF*–*phoA* sandwich fusions *in vitro* by using the *Pvu* II (bp 297) and *Bss*HII (bp 1059) sites of *malF*. The fusion joints are in regions that code for the second and third periplasmic domains of MalF, respectively. The fusion joints are very close to the previously isolated *malF*–Tn*phoA* fusions E (bp 296) and N (bp 1051) (7). As with these Tn*phoA* fusions, the two sandwich fusions had high specific AP

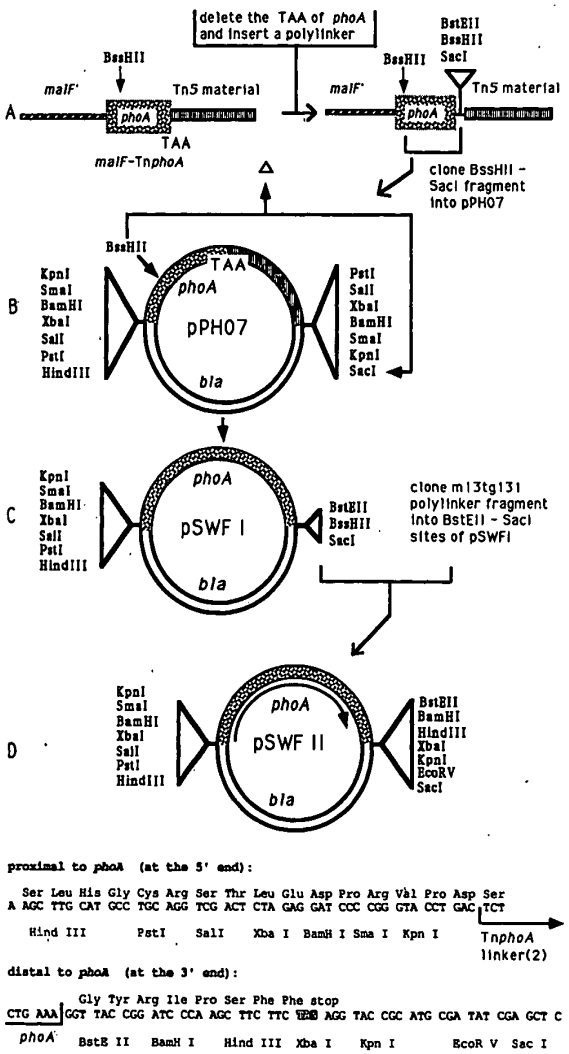
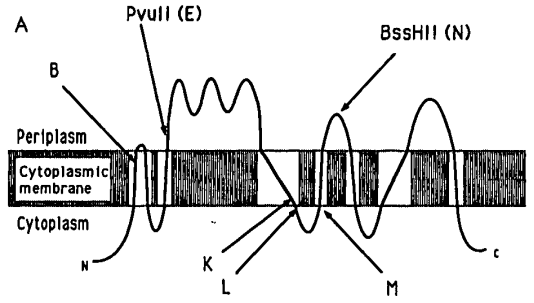


FIG. 2. The construction of the *phoA* sandwich fusion cassette pSWFII. pSWFII was made by inserting a polylinker at the 3′ end of *phoA* between the last codon of *phoA* and its stop codon, using the Tn*phoA* cassette pPHO7. (*A*) Starting with *malF*–Tn*phoA* fusion B (7), the stop codon of *phoA* was removed and a polylinker containing *Bst*EII, *Bss*HII, and *Sac* I sites was introduced using an oligonucleotide. (*B* and *C*) A 1-kbp *Bss*HII–*Sac* I fragment of *malF*–Tn*phoA* was exchanged with a 2.3-kbp *Bss*HII–*Sac* I fragment of Tn*phoA* cassette pPHO7 (5), yielding pSWFI. (*D*) The polylinker of pSWFI was extended by cloning parts of the phage m13tg131 polylinker into the *Bst*EII–*Sac* I sites of the pSWFI linker. The linker regions of pSWFII are shown below the plasmid diagram.



B

Fusion-joints	TnphoA fusions Multi copy	Single copy	sandwich fusions Multi copy	Single copy
B	1345	30.8	970	6.5
*PvuII (E)	1190	(30)	631	7.0
K	627	8.7	6	<0.1
L	723	17.5	24	0.1
M	230	2.4	5	<0.1
*BSSHII (N)	735	(26)	302	2.7

* these fusions were constructed using pSWFII.

FIG. 3. AP activities of *malF′–phoA–′malF* fusion and *malF′*–Tn*phoA* fusion strains. (*A*) The location of the fusion joints of *malF*–Tn*phoA* and *malF′–phoA–′malF* sandwich fusions used in this study. Each arrow points to the site at which the fusion joint is located in the topological model of MalF. The fusions were present on a multicopy plasmid. The exact fusion joints were as follows: B at bp 94, *Pvu* II (E) at bp 297, K at bp 907, L at bp 921, M at bp 958, and *Bss*HII (N) at bp 1059. (*B*) Comparison of the AP activities of Tn*phoA* and sandwich fusions. Fusions were present on a multicopy plasmid or in single copy inserted into the chromosome. Tn*phoA* fusions were inserted at the *phoA* locus as described (7). Values for the E and N fusions in parentheses are from previously published data (7). Single-copy data have been corrected by subtracting a background value (0.1 unit) measured in the *phoA*-deletion strain DHB4.

activities (Fig. 3). Thus, tethering AP at both its N and C termini does not severely interfere with the enzymatic activity of the protein.

Conversion of *malF*–Tn*phoA* Fusions to *malF'–phoA–'malF* Sandwich Fusions. Since sandwich fusions to periplasmic domains of MalF exhibited AP activities similar to those of related Tn*phoA* fusions obtained earlier, we wished to see whether sandwich fusions to cytoplasmic domains would display different properties. As we have described (7), *malF–phoA* fusions to the beginning of the third cytoplasmic domain of MalF (K and L) export almost as much AP to the periplasm as *phoA* fusions to the preceding and following periplasmic domain of MalF. A fusion to the end of the same cytoplasmic domain (M) exported much less AP, showing about 10 times lower AP activity. We suggested that C-terminal information might be necessary to determine the correct topology of the hybrid protein. To test this hypothesis, we have taken the K, L, and M fusions and, in a series of steps, restored the missing C-terminal portions of MalF. The same procedure was used with one of the previously obtained periplasmic fusions, B. In this way, we converted the *malF*–Tn*phoA* fusions B, K, L, and M to *malF'–phoA–'malF* sandwich fusions. Subsequently, the final constructs (Fig. 1*E*) were tested for the amount, the correct size, and the stability of hybrid protein made as well as for their AP activities.

The AP activities of sandwich fusions to the first, second, and third periplasmic domains were almost as high as those of Tn*phoA* fusions to the same domains (Fig. 3). The sandwich fusions derived from the K, L, and M fusions, all having cytoplasmic fusions joints, exhibited very low AP activities. This is in contrast to the activities seen with the original Tn*phoA* fusions. In particular, the K and L sandwich fusions gave activities 30–100 times lower than the corresponding Tn*phoA*-obtained fusions. The same difference in activities was obtained after transfer of the sandwich fusions and Tn*phoA* fusions in single copy onto the chromosome (Fig. 3). The low AP activity of sandwich fusions K, L, and M are not due to the breakdown of the hybrid proteins (unpublished data). Thus, it appears that we were able to stabilize the cytoplasmic location of the AP in the K and L fusions by adding back the C-terminal part of MalF that had been deleted in the Tn*phoA* fusions to the same base pair of *malF*.

Size, Stability, and Amount of Sandwich Fusion Proteins Made. The size, stability, and amount of sandwich fusion proteins made were tested by doing pulse–chase experiments. To be able to detect AP as well as MalF breakdown products, we used polyclonal antibodies raised against a MalF–PhoA fusion protein. The antibodies recognize various MalF–PhoA fusion proteins, wild-type AP, and wild-type MalF (B. Traxler and J.B., unpublished results).

Since all sandwich fusions contained the complete MalF and AP sequences, they all exhibited the same size in 10% polyacrylamide gels (data not shown). They migrated at about 95 kDa, whereas their predicted molecular mass is about 106 kDa. The difference between predicted and observed molecular mass is probably due to the hydrophobic character of MalF. Wild-type MalF itself migrates at about 17 kDa lower than its actual molecular mass in the same type of gel (22). The difference sites of insertion of AP within MalF apparently did not influence the behavior of the hybrid proteins in polyacrylamide gels.

All six hybrid proteins tested showed little breakdown during 30 min of chase. We could not detect any breakdown products (see Fig. 4). The amount of sandwich fusion proteins made was a littl lower than that of Tn*phoA* fusions (data n t sh wn), which is reflected in the observed lower AP activities (Fig. 3). It was also observed that after about 15 min of induction of the *tac* promoter with 10 mM isopropyl

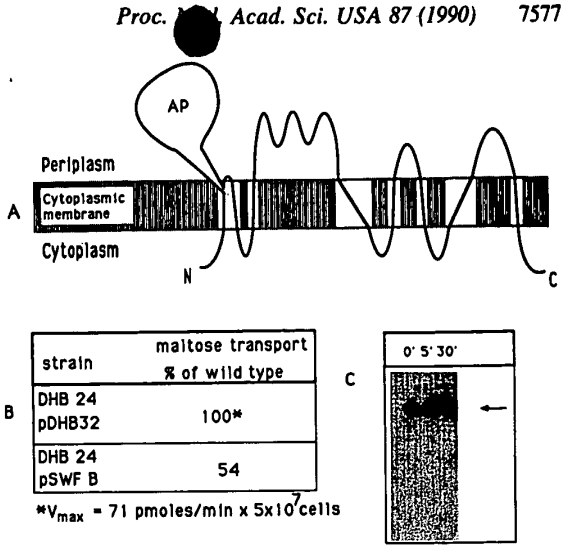


FIG. 4. A bifunctional sandwich fusion. (*A*) Proposed topology of the bifunctional protein encoded by the sandwich fusion *malF'–phoA–'malF* B. (*B*) Maltose transport activity of *malF'–phoA–'malF* sandwich fusion B. For maltose transport assays cells were grown to midlogarithmic phase in M63/0.2% glycerol/0.2% maltose. [^{14}C]-Maltose was applied at a concentration of 6.5 μM. (*C*) The stability of the sandwich fusion protein B examined in a pulse–chase experiment. Cells growing exponentially in M63/0.2% glycerol were labeled with [^{35}S]methionine for 2 min. Zero, 5, and 30 min after the addition of 25 mM L-methionine the cells were lysed and the proteins were precipitated using antibodies that recognize AP as well as MalF.

β-D-thiogalactoside, the rate of *de novo* synthesis of the hybrid proteins began to decrease (data not shown).

A Bifunctional Sandwich Fusion. Cells carrying the *malF'–phoA–'malF* fusion B to bp 94 of *malF* were both AP$^+$ (Fig. 3) and MalF$^+$ (Fig. 4). The cells were able to transport maltose effectively (54% of wild-type rates). The high transport activity of these cells was not due to the overproduction of the sandwich fusion protein. The amount of MalF and AP crossreacting material produced in the strain used in the transport assay was less than is seen from a wild-type chromosomal *malF* gene (data not shown). Since we could not detect any breakdown of the hybrid protein, we believe that both AP and MalF activities are exhibited by the sandwich fusion protein itself. The finding that the sandwich fusion protein B is able to transport maltose indicates that the C terminus of the hybrid protein must have inserted into the membrane with the correct topology.

DISCUSSION

We have shown that when AP of *E. coli* is tethered at both its N and C termini to another protein, it retains its enzymatic activity. We have inserted AP in each of three periplasmic domains of the multispanning cytoplasmic membrane protein MalF. These sandwich fusion proteins exhibit high specific AP activity comparable to that of MalF–PhoA fusion proteins obtained with Tn*phoA*, in which a C terminal portion of MalF is missing. The high levels of activity of the sandwich fusion proteins are somewhat surprising, since AP must dimerize in order to be enzymatically active (23). One might have thought that fusing the protein at both ends would interfere with this process. Our findings provide further t stimony to the extent to which proteins can be manipulated without interfering with their activity.

We have also constructed sandwich fusions in which AP is inserted in a cytoplasmic domain of MalF. These were derived from the previously isolated Tn*phoA* fusions in the same domain, which are missing the C terminus of MalF. The sandwich fusions showed 30–100 times lower enzymatic

activity than the original fusions from which they were construct d. The lower activity is an indication that the AP is predominantly localized to the cytoplasm. In contrast, the original fusion strains, in which the C terminus of MalF was missing, exported considerable amounts of AP to the periplasm. These results suggest that the presence of the C terminus of MalF in the sandwich fusion protein stabilizes the cytoplasmic location of AP.

The stabilization of AP to the cytoplasm in these fusions may be due to the proper assembly of the C-terminal portion of MalF into the membrane. With both the N- and C-terminal portions of MalF assuming their native conformation in the membrane, the inserted AP would be firmly held in the cytoplasm. That C-terminal portions of MalF can assemble correctly in sandwich fusions is indicated by our finding that the B sandwich fusion retains MalF function (see below). Our current hypothesis is that topology is initially determined by local effects of hydrophobicity and charge (7). If this were the case, we would expect that the C terminus of sandwich fusions would, in most cases, be correctly inserted in the membrane. The interactions that result in the appropriate localization of AP in fusions to cytoplasmic domains would be the same interactions that stabilize the native protein.

Our results indicate that the use of sandwich fusions for analyzing the arrangement of proteins in the membrane provides results that are better correlated with actual topology than those obtained with Tn*phoA* fusions. It appears that sandwich fusions to cytoplasmic domains may always show AP activities 20 times or more lower than fusions to periplasmic domains. This approach could be particularly useful when studies with the Tn*phoA* fusions yield ambiguous results.

We have shown in this study that it is possible to isolate bifunctional sandwich fusions. Cells carrying the *malF'–phoA–'malF* fusion B exhibited both AP and MalF activity. During purification of this hybrid protein any detectable AP activity was strictly correlated with the presence of the sandwich fusion protein (B. Traxler and J.B., unpublished results). This indicates that the hybrid protein is stable and is most likely itself responsible for both activities. (We can only assume this for the MalF activity, since it is not easy to monitor. The reconstitution of the multicomponent maltose transport system with separately purified components has not as yet been reported.) We also do not know whether all of the fusion protein is bifunctional or whether one subpopulation of the hybrid molecules is responsible for AP activity and another for MalF activity. Our results indicate that the insertion of the large AP molecule does not disrupt the structure of MalF in this fusion protein. We have recently shown that deletions of the first transmembrane segment of MalF also produce a protein that functions in maltose transport. Thus, this N-terminal segment of MalF is not required for its function.

The sandwich fusions make slightly less protein than the corresponding Tn*phoA* fusions. We have observed that high levels of production of the MalF'–PhoA–'MalF hybrid proteins interfere with cell growth, often leading to cell death. Fifteen minutes after induction of the sandwich fusion genes the cells show reduced protein synthesis. These effects could explain the lesser synthesis seen with sandwich fusions.

Sandwich fusions may have uses besides the study of topology: for example, the AP hybrid proteins could be used for the production of antibodies against prot ins that are hard to purify. Since the entire target protein is present in the sandwich fusions, such antibody preparations may be directed against epitopes throughout the protein. *phoA* sandwich fusions could be used to study the export of proteins that pass through the outer membrane and are released from the cells, such as proteases or bacteria toxins. If inserted AP can also be exported to the medium, then halos of blue color should be seen with colonies plated on medium containing the 5-bromo-4-chloro-3-indolyl phosphate. This would provide a convenient phenotype for genetic studies. Sandwich fusions may also be used to construct nonpolar insertion mutations.

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Appendix C

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Appendix D

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Vol. 172, No. 3

Properties of New *Escherichia coli* Hfr Strains Constructed by Integration of pSC101-Derived Conjugative Plasmids

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Conjugative temperature-sensitive plasmids were derived from pSC101. These plasmids are useful in genetic analysis for two reasons: (i) they render possible the construction of new Hfr lines by plasmid integration at predetermined chromosomal loci via Tn*10* inverse transposition, and (ii) the Hfr characters are transducible via bacteriophage P1. We also showed that replication from pSC101 origin is deleterious for the plasmid-chromosome fusion.

Techniques based on bacterial conjugation remain extremely useful for genetic analysis and the manipulation of the *Escherichia coli* genome. Use of the classical sex factor F is, however, limited by the low number of integration sites (20) and the absence of an easy system with which to convert a female strain into a given Hfr type. These limitations result from the usual mode of F integration, by recombination between insertion sequences (18), and from the large size of this plasmid (close to 100 kilobases [kb] [26]), which renders conversion to a given Hfr type by bacteriophage P1-mediated transduction difficult. To cope with these limitations, we have constructed "pseudo-F" plasmids with the following properties: (i) efficient autotransfer; (ii) conditional replication (to make their integration into the chromosome necessary for maintenance under nonpermissive conditions); (iii) antibiotic resistance (to enable selection for plasmid maintenance); and (iv) relatively small size (to insure transducibility of the integrated plasmids by phage P1-mediated transduction). We have been able to direct integration of these plasmids into predetermined chromosomal positions, and we present here a series of new transducible Hfr types. In addition, we have observed that, once they are integrated into the chromosome, these plasmids are deleterious to the host when placed in permissive conditions for plasmid replication. We have shown that this phenotype is a direct consequence of the activity of the pseudo-F replication system, which comes from a temperature-sensitive replication mutant of pSC101.

MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmid pOX38 is the larger *Hin*dIII fragment of plasmid F, which contains the *tra* operon and the *rep* region (13). Plasmid pHO1 (12) harbors the 2.9-kb autoreplicative *Hin*cII fragment of pHS1, a temperature-sensitive derivative of pSC101 (16). Plasmids pHP45-ΩAp and pHP45-ΩCm, the sources for resistance markers, were described by Fellay et al. (10). Plasmid pUCAG is derived from pUC18 by cloning of a synthetic fragment harboring two pSC101 RepA boxes (L. Caro, personal communication). Bacteriophage P1L4 is from our collection. Strain C600 r^+ m^- (F^- *hsdM thr leu thi lac tonA supE*, our collection) was the primary recipient of the two plasmids described here, pVF9 and pVF10 (strains LN1916 and LN1950, respectively). Strain CB0129 is F^- W1485 *thy leu thi supE* (our collection). This strain received the Tn*10* insertions described in Table 1 via P1 transduction. The female strain used routinely as recipient in matings, LN2338, is W945 *xyl mtl ile metA* or B *ara lacYI purE gal pyrC76*::Tn*10 trp his gyrA* (Nal^r) *argG thi rpsL* (Sm^r).

Media and enzymes. Luria complex medium and Vogel and Bonner E minimal medium were used as previously described (19). Various antibiotics were added at the following concentrations: ampicillin, 75 μg/μl; chloramphenicol, 20 μg/μl; fusaric acid, 12 μg/μl; nalidixic acid, 40 μg/μl; tetracycline, 50 μg/μl; and streptomycin, 160 μg/μl. Restriction endonucleases and T4 DNA ligase were used as recommended by the manufacturer (GIBCO/Bethesda Research Laboratories, Gaithersburg, Md.).

Genetic procedures. (i) In vivo. P1-mediated transductions and conjugations were performed as previously described (19). Gradients of marker transmission were established as follows: one male per five LN2338 females mixed in Luria broth (at about 2×10^8 bacteria per ml) and incubated at 37°C for 2 h without agitation. The bacteria were then plated at the desired dilutions on selective plates with nalidixic acid for male contraselection and inhibition of conjugational transfer after plating. The search for Tc^s derivatives of Tn*10*-carrying strains was conducted as described by Maloy and Nunn (21).

(ii) In vitro. Methods for preparation of plasmid DNA, enzymatic digestion, purification of DNA fragments from preparative agarose gels, DNA ligation, Southern analysis and physical mapping were as previously described (19).

RESULTS

Construction of plasmids pVF9 and pVF10. To construc small, autotransferable, temperature-sensitive, and antib otic resistance-carrying plasmids, we ligated together (i) 43.2-kb *Bam*HI fragment of pOX38 which carries the *tr* operon of the F plasmid (26); (ii) a 2.9-kb *Bam*HI fragme carrying the *rep* and *par* regions of a temperature-sensiti replication mutant of pSC101 (12); and (iii) either a 3.6-k *Bam*HI fragment or a 2-kb *Bam*HI fragment carrying chloramphenicol or an ampicillin resistance determinar respectively (these latter fragments were prepared fr resistance "interposons" described by Fellay et al. [10 The construction of the two pseudo-F plasmids, pVF9 (Cm and pVF10 (Ap^r), is diagrammed in Fig. 1. To our knov edge, the two plasmids are devoid of any active inserti

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TABLE 1. Pseudo-Hfr strains[a]

Strain	Integrated plasmid	Integration site (min)	Direction of transfer[b]	Cryosensitivity factor[c]
LN1946	pVF9	*trp*::Tn*10* (27)	+	0.3
LN1948	pVF9	*zdf*::Tn*10* (35)	+	10^{-3}
LN1967	pVF9	*zda*::Tn*10* (30)	+	0.2
LN1973	pVF9	*malE*::Tn*10* (91)	−	3×10^{-4}
LN1974	pVF9	*malE*::Tn*10* (91)	−	1
LN1975	pVF10	*malE*::Tn*10* (91)	−	0.2
LN1980	pVF9	*pyrC*::Tn*10* (23)	+	0.5
LN2061	pVF9	*metE*::Tn*10* (89)	−	1
LN2062	pVF9	*metE*::Tn*10* (89)	+	0.2
LN2080	pVF9	*metE*::Tn*10* (89)	+	0.1
LN2085	pVF10	*trp*::Tn*10* (27)	−	0.7
LN2275	pVF9	*ptsM*::Tn*10* (40)	−	1

[a] All pseudo-Hfr characters presented in this table resulted from inverse transposition into Tn*10* insertions and are transducible by phage P1.
[b] +, Clockwise; −, counterclockwise.
[c] Ratio of plating efficiency at 30°C to that at 43°C.

sequences, although it should be noted that the pSC101-derived fragment contains a part of IS102 (4).

Selection for an Hfr state. The transferable plasmids pVF9 and pVF10 are stable at 30°C but are lost at temperatures above 37°C. Upon incubation at 43°C in the presence of the relevant drug, antibiotic-resistant clones appear at a frequency close to 10^{-4}. These clones presumably resulted from multiplication of bacteria harboring an integrated pseudo-F plasmid. In a study performed on the pVF9-carrying strain LN1916, we found that, among 106 independent isolates on chloramphenicol-containing medium incubated at 43°C, 83 displayed Hfr properties, i.e., a capability to transfer chromosomal markers at a high rate to recipient females. The non-Hfr clones were probably due to an integration event disrupting the *tra* operon. These events were rarer than expected, considering that the *tra* operon (33 kb) constitutes about 70% of the potential target for plasmid integration. pVF9 integration sites were widely dispersed along the chromosome in the pseudo-Hfr strains (Fig. 2). No attempt has been made to further characterize the chromosomal sequences involved in pVF9 integration.

In order to direct plasmid integration to predetermined loci, we have exploited inverse transposition (7, 15) of pVF9 or pVF10 into a chromosomal Tn*10* insertion. In this transposition event, diagrammed in Fig. 3, which involves the inside ends of the Tn*10*-associated IS*10*'s, the internal part of Tn*10* is replaced by the target DNA. The Tn*10*-associated tetracycline resistance character (Tc^r) is lost, and consequently the bacteria become resistant to fusaric acid (Fa^r) (5). By associating selection for maintenance of the pseudo-F resistance character at 43°C together with acquisition of a Fa^r character, we have constructed a series of Hfr strains derived by pseudo-F integration by Tn*10* inverse transposition (Table 1). The frequency of integration by inverse transposition cannot be precisely determined, since the procedure involves an enrichment for Tc^s clones rather than direct selection. The mode of integration has been investigated by Southern analysis for six different Tc^s Hfr strains. All six displayed IS*10* copies at the chromosome-plasmid junctions (data not shown). Both orientations of the integrated plasmid were observed at some loci (Table 1). Taking advantage of the many available Tn*10* insertions (2, 24), this procedure makes it possible to undertake the construction of

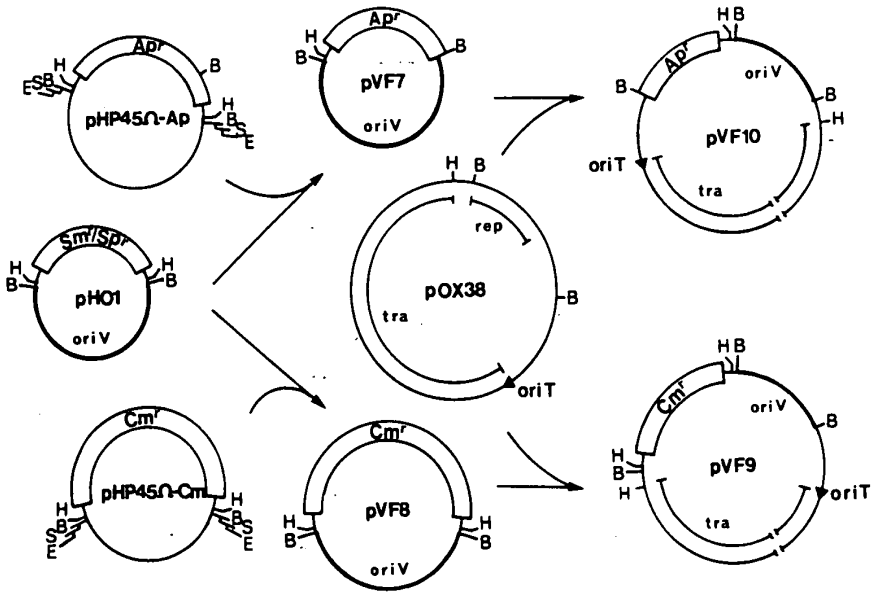

FIG. 1. Construction of plasmids pVF9 and pVF10. In a first step, the *Bam*HI replicative fragment of plasmid pHO1 was purified, dephosphorylated, and ligated with total digests by *Bam*HI endonuclease of plasmid pHP45-ΩCm or pHP45-ΩAp. After transformation of a C600 r⁻ m⁺ strain, Cm^r and Ap^r transformants were selected and tested for temperature sensitivity. Plasmids pVF7 and pVF8 were retained after restriction analysis of a number of transformants of Ts plasmids. In a second step, plasmids pVF7 and pVF8 were partially digested by *Bam*HI. Linear unit-size DNA was purified, dephosphorylated, and ligated to the large purified *Bam*HI fragment of pOX38. After transformation and selection of Cm^r and Ap^r at 30°C, these transformants were screened for transfer and temperature-sensitive maintenance of the resistance character. Plasmids pVF9 and pVF10 were retained after restriction analysis. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sma*I; *oriT*, F origin of transfer; *oriV*, the partition and replication region of plasmid pHO1 (not oriented); *rep*, the maintenance and replication region of plasmid F; *tra*, F transfer operon.

Appendix E

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Isolation of the Bacteriophage Lambda Receptor from *Escherichia coli*

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A factor which inactivates the phage lambda can be extracted from *Escherichia coli*. This factor is a protein and is located in the outer membrane of the bacterial envelope. It is found in extracts of strains which are sensitive to phage lambda, but not in extracts of strains specifically resistant to this phage. We conclude that this factor is the lambda receptor, responsible for the specific adsorption of the phage lambda to *E. coli* cells. A partial purification of the lambda receptor is described. Inactivation of the phage by purified receptor is shown to be accompanied by the release of deoxyribonucleic acid from the phage.

The cell envelope of *Escherichia coli* is a complex, multilayered structure composed of the cytoplasmic membrane and the cell wall which itself includes the peptidoglycan layer and the outer or L membrane. Little is known about the morphology or the function of the outer membrane. It was considered by some workers as a penetration barrier and its study was approached through the analysis of mutants with altered antibiotic sensitivity (2, 5, 9, 11, 16). Another approach is the investigation of receptor molecules located on the bacterial surface. Receptors for colicin E (12) and colicin K (12, 21) have been identified as molecules containing protein moieties. Receptors for phage T2 and T6 have also been demonstrated to involve a protein moiety, whereas T3, T4, T7, and C21 adsorb to lipopolysaccharide components of the outer membrane (20).

The isolation of the receptor for phage lambda, reported here, is another example of this type of approach. The purification and characterization of this receptor should yield information on the structure and function of the bacterial cell envelope. Also, it will provide a means to study the mechanism of phage adsorption and other early steps in the phage-host interaction.

The genetics of the lambda receptor is well documented. It has been demonstrated that all mutations specifically impairing adsorption of phage λ inactivate a single cistron, *lam B* (80.5 min on the genetic map of *E. coli*) (17; and M. Hofnung, Ph.D. thesis, Paris, 1972). This cistron is in one of the operons involved in maltose metabolism. Therefore, *lam B* can be inactivated either directly by mutations inside the gene, or indirectly by mutations in other genes of the maltose system. About 20% of all resistant mutations map inside *lam B* and yield λr Mal$^+$ phenotype. The remaining 80% of λr mutants are Mal$^-$. Most of these have a mutation in *mal T* (66 min), the positive regulator of the maltose system. Polar mutations in *mal K*, a maltose permease gene located in the same operon as *lam B*, also yield a λr Mal$^-$ phenotype. There are no host range mutants of lambda which infect any λr Mal$^-$ strain. However, host range mutants can be selected which grow on some λr Mal$^+$ *lam B* mutants. This may be because λr Mal$^-$ bacteria contain no *lam B* gene product, whereas some λr Mal$^+$ mutants may have a modified gene product which allows the adsorption of phage which have a mutant host range. The *lam B* gene may code for an enzyme involved in the synthesis of lambda receptors or it may code for a structural component of the receptor itself. The work presented here utilizes λ-resistant mutants to correlate the receptor with a protein which is solubilized from the outer membrane and which inactivates phage lambda. The partial purification and characterization of this protein is discussed.

MATERIALS AND METHODS

Abbreviations and definitions. Abbreviations used are as follows: PFU, plaque-forming units; MOI, multiplicity of infection; λr and λs, resistant and

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Institut Pasteur, Paris, France

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LS AND METHODS

d definitions. Abbreviations
FU, plaque-forming units; MOI,
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sensitive to phage λ, respectively; Mal⁺ and Mal⁻, able and unable to use maltose as a carbon source, respectively; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; Tris buffer, tris(hydroxymethyl)aminomethane (pH was adjusted with HCl). Room temperature ranged from 22 to 24 C.

QAE-Sephadex, an anionic exchange resin, was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Radioactive isotopes were obtained from Commissariat à l'Energie Atomique, France. Sodium cholate was purchased from Schuchardt München, Germany.

Media and bacterial strains. The minimal medium (M63) used for growth and the complete medium used for plating phage were described elsewhere (15). Strain HfrG6 (15) is the wild-type λsMal⁺ strain. Pop 1730 is a spontaneous λrMal⁻ mutant of HfrG6; it carries the deletion MBΔ17 covering part of the gene *mal K* and probably part of the gene *lam B* (M. Hofnung, D. Hatfield, and M. Schwartz, J. Bacteriol., in press). The λrMal⁺ strain CR63 (1) is the standard host for host range mutants of λ. Several other strains are used only in the experiments described in Table 2 and Fig. 2; their genotype is given in the legends.

Phage λV is a virulent mutant of λ⁺ (8). The phage λVh, a spontaneous host range mutant of λV, was isolated in this laboratory by plating λV on strain CR63. In the text λV is referred to simply as λ and λVh as λh.

The indicator strain used for plating phage was HfrG6.

Assay of receptor activity. Unless otherwise indicated, inactivation of λh was assayed in the absence of $CHCl_3$ as described here. One milliliter of a phage stock containing between 3×10^3 and 6×10^3 PFU in 10^{-2} M $MgSO_4$ was mixed with 1 ml of cholate-EDTA extract (see below) diluted as given for each experiment in 10^{-2} M Tris buffer (pH 7.5). In the control experiments the extract was replaced by Tris buffer. The mixture was incubated at 37 C. At times as indicated, samples of 0.2 ml were taken and added to tubes, kept at 37 C, containing 10^9 indicator bacteria in 0.1 ml 10^{-2} M $MgSO_4$. After an incubation of 5 min to allow adsorption, 3 ml of soft agar was added to each tube and the contents were plated.

Assay of receptor activity in the presence of $CHCl_3$. Purified λh, and to a lesser extent, purified λ, are sensitive to chloroform. Efficient protection against the inactivation of those phages by chloroform is obtained by adding BSA to the phage suspension. Hence the phage stocks used in these experiments were diluted in 10^{-2} M $MgSO_4$ containing 200 μg of BSA per ml. One milliliter of such a phage dilution containing between 3×10^5 and 6×10^5 PFU was mixed with 1 ml of a dilution of cholate-EDTA extract. About 10 drops of $CHCl_3$ were added, and the mixture was shaken vigorously. At the times indicated 0.2-ml portions were transferred into 10 ml of 10^{-2} M $MgSO_4$, and 0.1 ml of those dilutions were plated with indicator bacteria.

Extraction of receptor. Two liters of *E. coli* HfrG6 cells were grown at 37 C in minimal medium containing 0.02% histidine and 0.4% maltose. The bacteria were harvested in exponential phase at an optical density of 1 A_{600}/ml (5×10^8 cells/ml), centrifuged, and suspended in 100 ml of 1.0% cholate, 2×10^{-3} M EDTA, 10^{-2} M Tris buffer, pH 7.5. After shaking 30 min at 37 C, the suspension was centrifuged at 20,000 × *g* for 40 min, and then dialyzed versus 2% cholate, 10^{-2} M Tris buffer (pH 7.5). Protein concentration was determined by the method of Lowry et al. (10), using BSA as a standard.

Chloroform-methanol treatment. Two volumes of $CHCl_3$ and 1 volume of CH_3OH were added per volume of extract, and the mixture was stirred at room temperature for 5 min. The phases were separated by low-speed centrifugation. The precipitate at the interface was carefully removed, suspended in 10 ml of 2% cholate-10^{-2} M Tris buffer (pH 7.5), and dialyzed versus the same buffer to remove remaining CH_3OH and $CHCl_3$. The dialysis fluid was then changed to 10^{-2} M Tris (pH 7.5).

Column chromatography. Routinely, 15 ml of extract, at a protein concentration of approximately 3 mg/ml, was applied to a QAE-Sephadex column (30 cm by 1.5 cm) equilibrated with 10^{-2} M Tris buffer (pH 7.5). The column was eluted at 6 ml/h with a 300-ml salt gradient from 0 to 0.8 M NaCl in Tris buffer, followed by a detergent-salt wash (2% cholate, 1.5 M NaCl in 10^{-2} M Tris, pH 7.5). Ninety-seven percent of the protein applied, but no receptor activity, was washed off the column by the salt gradient. All detectable receptor activity was found in the cholate wash. Fractions containing the activity were pooled and dialyzed versus 10^{-2} M Tris buffer (pH 7.5) and then applied to a second QAE-Sephadex column (25 cm by 0.8 cm). Elution was with a 100-ml gradient from 0 to 0.6 M NaCl in 2% cholate, 10^{-2} M Tris buffer (pH 7.5). The fractions containing receptor activity were pooled and dialyzed versus 1% cholate, 10^{-2} M Tris buffer (pH 7.5). The purification is summarized in Table 1.

^{3}H- and ^{14}C-labeled bacteria. Labeled bacteria

TABLE 1. *Purification of the lambda receptor*[a]

Purification step	Sp act	Recovery (%)
Cholate-EDTA extraction	1	100
$CHCl_3$:CH_3OH treatment	1	100
1st QAE-Sephadex column	11	39
2nd QAE-Sephadex column	73	34

[a] Conditions for each step in purification are given in detail in Materials and Methods. The specific activity is expressed relative to that observed in the crude cholate-EDTA extract. It is calculated as follows: the rate constant K (s^{-1}) is determined for each fraction (see legend to Fig. 1), multiplied by the appropriate factor to account for differences in volume between the various fractions and the crude extract, and divided by the total protein content of the given fraction (in the purification given here protein content was calculated from ^{3}H-leucine present in each fraction).